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Communication

An assessment of three human methylenetetrahydrofolate dehydrogenase/cyclohydrolase ligand complexes following further refinement.

Renata V. Bueno, Alice Dawson and William N. Hunter

Division of Biological Chemistry and Drug Discovery, College of Life Sciences, University of Dundee, Dundee, DD1 5EH, Scotland (UK)

Correspondence e-mail: w.n.hunter@dundee.ac.uk

Synopsis

Further refinement of three methylenetetrahydrofolate dehydrogenase/cyclohydrolase antifolate inhibitor complexes in the Protein Data Bank has produced models that allow for a critical reassessment of ligand placement. One complex has a well-ordered ligand in a catalytic site and the model provides an improved description of enzyme-inhibitor interactions. One ligand may adopt two conformations in the binding site rather than the single one previously described. There is no evidence to support incorporation of the third compound in the model. Our interpretation of the data supports a correlation between the models and inhibition activity for two of the compounds. In the case of the third, inconsistencies are noted that would need to be addressed by further work.

Abstract

Enzymes involved in folate metabolism are key drug targets for cell growth modulation and accurate crystallographic structures provide templates to be exploited for structure-based ligand design. In this context, three ternary complex structures of human methylenetetrahydrofolate dehydrogenase/cyclohydrolase have been published and potentially represent starting points for development of new antifolate inhibitors. However, our inspection of the models and deposited data revealed deficiencies and raised questions about the validity of the structures. A number of inconsistencies relating to the publication were also identified. We carried out additional refinement with the deposited data, seeking to improve the models and to then validate the complex structures or correct the record. In one case, we support the inclusion of the inhibitor in the structure and alterations to the model allow us to describe details of enzyme-ligand interactions not previously discussed. For one weak inhibitor, the data suggest that the ligand may adopt two poses in the binding site both with few interactions to the enzyme. In the third case, that of a potent inhibitor, we note inconsistencies in the assignment of the chemical structure and no evidence to support inclusion of the ligand in the active site.

Keywords: methylene-tetrahydrofolate dehydrogenase, methenyl-tetrahydrofolate cyclohydrolase, antifolates, structure-based ligand design.

PDB codes: 6ECP, 6ECQ, 6ECR.

1.0 Introduction

Methylene-tetrahydrofolate dehydrogenase, methenyl-tetrahydrofolate cyclohydrolase and 10-formyl-tetrahydrofolate synthetase support the inter-conversion of tetrahydrofolate derivatives, directing one-carbon donor units to biosynthesis of amino acids and nucleotides {ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1021/bi971906t", "ISBN" : "0006-2960", "ISSN" : "00062960", "PMID" : "9454603", "abstract" : "The kinetic properties of three methylenetetrahydrofolate dehydrogenase-cyclohydrolase (D/C) enzymes (the NADP-dependent bifunctional domain of the human cytoplasmic trifunctional enzyme, the human mitochondrial NAD-dependent bifunctional enzyme, and the NAD(P)-dependent bifunctional enzyme from Photobacterium phosphoreum) were determined in both forward and reverse directions. In the forward direction, the enzymes possess widely different ratios of kcat C/Kcat D, but all channel methenylH4folate produced by the D activity to the C activity with approximately the same efficiency. A deuterium isotope effect is observed with the human NADP-dependent enzyme in both forward and reverse dehydrogenase assays, consistent with hydride transfer being rate limiting for the interconversion of methenyl- and methyleneH4folate. However, no kinetic isotope effect is observed for the overall reverse reaction (formylH4folate to methyleneH4folate). We devised an assay to measure the reverse cyclohydrolase activity independent of the dehydrogenase, and determined that the Kcat (overall reverse) for each enzyme is approximately equal to the Kcat for its reverse cyclohydrolase activity. Therefore, the rate-limiting step in the overall reverse reaction is not hydride transfer by the dehydrogenase, but the production of methenylH4folate catalyzed by the cyclohydrolase. The reverse cyclohydrolase activities of the NADP-dependent D/C and the P. phosphoreum enzymes, but not the mitochondrial NAD-dependent enzyme, can be stimulated 2-fold by the addition of 2',5'-ADP. The results suggest that the cyclohydrolases of the human NADP dependent and P. phosphoreum enzymes are optimized to catalyze the reverse reaction in the presence of bound coenzyme. These results imply that essentially all of the methenylH4folate produced by the cyclohydrolase in the reverse reaction is channeled to the dehydrogenase.", "author" : [{ "dropping-particle" : "", "family" : "Pawelek", "given" : "Peter D.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "MacKenzie", "given" : "Robert E.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "Biochemistry", "id" : "ITEM-1", "issue" : "4", "issued" : { "date-parts" : [["1998"]] }, "page" : "1109-1115", "title" : "Methenyltetrahydrofolate cyclohydrolase is rate limiting for the enzymatic conversion of 10-formyltetrahydrofolate to 5,10- methylenetetrahydrofolate in bifunctional

dehydrogenase-cyclohydrolase enzymes", "type" : "article-journal", "volume" : "37" }, "uris" : ["http://www.mendeley.com/documents/?uuid=c6033326-38e8-457b-af0e-22e8e505389d"] }], "mendeley" : { "formattedCitation" : "(Pawelek & MacKenzie, 1998)", "plainTextFormattedCitation" : "(Pawelek & MacKenzie, 1998)", "previouslyFormattedCitation" : "(Pawelek & MacKenzie, 1998)" }, "properties" : { "noteIndex" : 0 }, "schema" : "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }**}**. In humans, a trifunctional enzyme carries these activities. The enzyme comprises a C-terminal synthetase domain and an N-terminal domain possessing the dehydrogenase/cyclohydrolase (DHCH) activities **{**ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1016/S0969-2126(98)00019-7", "ISBN" : "0969-2126 (Print)\\r0969-2126 (Linking)", "ISSN" : "09692126", "PMID" : "9519408", "abstract" : "BACKGROUND: The interconversion of two major folate one-carbon donors occurs through the sequential activities of NAD(P)-dependent methylene[H4]folate dehydrogenase (D) and methenyl[H4]folate cyclohydrolase (C). These activities often coexist as part of a multifunctional enzyme and there are several lines of evidence suggesting that their substrates bind at overlapping sites. Little is known, however, about the nature of this site or the identity of the active-site residues for this enzyme family. RESULTS: We have determined, to 1.5 A resolution, the structure of a dimer of the D/C domain of the human trifunctional cytosolic enzyme with bound NADP cofactor, using the MAD technique. The D/C subunit is composed of two alpha/beta domains that assemble to form a wide cleft. The cleft walls are lined with highly conserved residues and NADP is bound along one wall. The NADP-binding domain has a Rossmann fold, characterized by a modified diphosphate-binding loop fingerprint-GXSXXXG. Dimerization occurs by antiparallel interaction of two NADP-binding domains. Superposition of the two subunits indicates domain motion occurs about a well-defined hinge region. CONCLUSIONS: Analysis of the structure suggests strongly that folate-binding sites for both activities are within the cleft, providing direct support for the proposed overlapping site model. The orientation of the nicotinamide ring suggests that in the dehydrogenase-catalyzed reaction hydride transfer occurs to the pro-R side of the ring. The identity of the cyclohydrolase active site is not obvious. We propose that a conserved motif-Tyr52-X-X-X-Lys56- and/or a Ser49-Gln100-Pro102 triplet have a role in this activity.", "author" : [{ "dropping-particle" : "", "family" : "Allaire", "given" : "M", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Li", "given" : "Y", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "MacKenzie", "given" : "R E", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Cygler", "given" : "M", "non-dropping-particle" : "", "parse-names" : false, "suffix" :

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"http://www.mendeley.com/documents/?uuid=1944f81b-4880-4cbc-b2d8-35515e3581fe"] }, { "id" : "ITEM-2", "itemData" : { "DOI" : "10.1111/febs.12025", "ISSN" : "1742464X", "PMID" : "23050773", "abstract" : "The bifunctional N(5),N(10)-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (DHCH or FolD), which is widely distributed in prokaryotes and eukaryotes, is involved in the biosynthesis of folate cofactors that are essential for growth and cellular development. The enzyme activities represent a potential antimicrobial drug target. We have characterized the kinetic properties of FolD from the Gram-negative pathogen Acinetobacter baumanni and determined high-resolution crystal structures of complexes with a cofactor and two potent inhibitors. The data reveal new details with respect to the molecular basis of catalysis and potent inhibition. A unexpected finding was that our crystallographic data revealed a different structure for LY374571 (an inhibitor studied as an antifolate) than that previously published. The implications of this observation are discussed.", "author" : [{ "dropping-particle" : "", "family" : "Eadsforth", "given" : "Thomas C.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "V.", "family" : "Maluf", "given" : "Fernando", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Hunter", "given" : "William N.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "FEBS Journal", "id" : "ITEM-2", "issue" : "23", "issued" : { "date-parts" : [["2012"]] }, "page" : "4350-4360", "title" : "Acinetobacter baumannii FolD ligand complexes - Potent inhibitors of folate metabolism and a re-evaluation of the structure of LY374571", "type" : "article-journal", "volume" : "279" }, "uris" : ["http://www.mendeley.com/documents/?uuid=39eaedb5-e306-4db7-8969-ab5098477c12"] }, { "id" : "ITEM-3", "itemData" : { "DOI" : "10.1371/journal.pone.0035973", "ISBN" : "1932-6203 (Electronic)\r1932-6203 (Linking)", "ISSN" : "1932-6203", "PMID" : "22558288", "abstract" : "<p>The bifunctional enzyme methylenetetrahydrofolate dehydrogenase \u2013 cyclohydrolase (FolD) is identified as a potential drug target in Gram-negative bacteria, in particular the troublesome <italic>Pseudomonas aeruginosa</italic>. In order to provide a comprehensive and realistic assessment of the potential of this target for drug discovery we generated a highly efficient recombinant protein production system and purification protocol, characterized the enzyme, carried out screening of two commercial compound libraries by differential scanning fluorimetry, developed a high-throughput enzyme assay and prosecuted a screening campaign against almost 80,000 compounds. The crystal structure of <italic>P. aeruginosa</italic> FolD was determined at 2.2 \u00c5 resolution and provided a template for an assessment of druggability and for modelling of ligand complexes as well as for comparisons with the human enzyme. New FolD inhibitors were identified and characterized but the weak levels of enzyme inhibition suggest

that these compounds are not optimal starting points for future development. Furthermore, the close similarity of the bacterial and human enzyme structures suggest that selective inhibition might be difficult to attain. In conclusion, although the preliminary biological data indicates that FolD represents a valuable target for the development of new antibacterial drugs, indeed spurred us to investigate it, our screening results and structural data suggest that this would be a difficult enzyme to target with respect to developing the appropriate lead molecules required to underpin a serious drug discovery effort.</p>", "author" : [{ "dropping-particle" : "", "family" : "Eadsforth", "given" : "Thomas C", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Gardiner", "given" : "Mary", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "V", "family" : "Maluf", "given" : "Fernando", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "McElroy", "given" : "Stuart", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "James", "given" : "Daniel", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Frearson", "given" : "Julie", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Gray", "given" : "David", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Hunter", "given" : "William N", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "PLoS ONE", "id" : "ITEM-3", "issue" : "4", "issued" : { "date-parts" : [["2012"]] }, "page" : "e35973", "title" : "Assessment of Pseudomonas aeruginosa N5,N10-Methylenetetrahydrofolate Dehydrogenase - Cyclohydrolase as a Potential Antibacterial Drug Target", "type" : "article-journal", "volume" : "7" }, "uris" : ["http://www.mendeley.com/documents/?uuid=a6e1a35f-2121-4613-889b-71a3ab57b2d0"] }], "mendeley" : { "formattedCitation" : "(Bermingham & Derrick, 2002; Eadsforth, Maluf et al., 2012; Eadsforth, Gardiner et al., 2012)", "plainTextFormattedCitation" : "(Bermingham & Derrick, 2002; Eadsforth, Maluf et al., 2012; Eadsforth, Gardiner et al., 2012)", "previouslyFormattedCitation" : "(Bermingham & Derrick, 2002; Eadsforth, Maluf et al., 2012; Eadsforth, Gardiner et al., 2012)" }, "properties" : { "noteIndex" : 0 }, "schema" : "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }**}**.

The structure of the human DHCH domain has been reported in complex with three inhibitors **{**ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1021/bi992734y", "ISBN" : "0006-2960 (Print)\\r0006-2960 (Linking)", "ISSN" : "00062960", "PMID" : "10828945", "abstract" : "Enzymes involved in tetrahydrofolate metabolism are of particular pharmaceutical interest, as their function is crucial for amino acid and

DNA biosynthesis. The crystal structure of the human cytosolic methylenetetrahydrofolate dehydrogenase/cyclohydrolase (DC301) domain of a trifunctional enzyme has been determined previously with a bound NADP cofactor. While the substrate binding site was identified to be localized in a deep and rather hydrophobic cleft at the interface between two protein domains, the unambiguous assignment of catalytic residues was not possible. We succeeded in determining the crystal structures of three ternary DC301/NADP/inhibitor complexes. Investigation of these structures followed by site-directed mutagenesis studies allowed identification of the amino acids involved in catalysis by both enzyme activities. The inhibitors bind close to Lys56 and Tyr52, residues of a strictly conserved motif for active sites in dehydrogenases. While Lys56 is in a good position for chemical interaction with the substrate analogue, Tyr52 was found stacking against the inhibitors' aromatic rings and hence seems to be more important for proper positioning of the ligand than for catalysis. Also, Ser49 and/or Cys147 were found to possibly act as an activator for water in the cyclohydrolase step. These and the other residues (Gln100 and Asp125), with which contacts are made, are strictly conserved in THF dehydrogenases. On the basis of structural and mutagenesis data, we propose a reaction mechanism for both activities, the dehydrogenase and the cyclohydrolase.", "author" : [{ "dropping-particle" : "", "family" : "Schmidt", "given" : "Andrea", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Wu", "given" : "Haiping", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Mackenzie", "given" : "Robert E.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Chen", "given" : "Victor J.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Bewly", "given" : "Jesse R.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Ray", "given" : "James E.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Toth", "given" : "John E.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Cygler", "given" : "Miroslaw", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "Biochemistry", "id" : "ITEM-1", "issue" : "21", "issued" : { "date-parts" : [["2000"]] }, "page" : "6325-6335", "title" : "Structures of three inhibitor complexes provide insight into the reaction mechanism of the human methylenetetrahydrofolate dehydrogenase/cyclohydrolase", "type" : "article-journal", "volume" : "39" }, "uris" : ["http://www.mendeley.com/documents/?uuid=f2912831-2d65-4e42-aac0-bed1b313204d"] }], "mendeley" : { "formattedCitation" : "(Schmidt et al., 2000)", "plainTextFormattedCitation" : "(Schmidt et al., 2000)", "previouslyFormattedCitation" : "(Schmidt et al., 2000)" }, "properties" : { "noteIndex" : 0 }, "schema" :

"https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }}, with coordinates and structure factors available in the Protein Data Bank (PDB). The structures of homologues, together with information on inhibitors have been exploited to inform early stage drug discovery targeting primitive eukaryotic parasites and bacteria {ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1111/febs.12025", "ISSN" : "1742464X", "PMID" : "23050773", "abstract" : "The bifunctional N(5),N(10)-methylenetetrahydrofolate dehydrogenase/cyclohydrolase (DHCH or FolD), which is widely distributed in prokaryotes and eukaryotes, is involved in the biosynthesis of folate cofactors that are essential for growth and cellular development. The enzyme activities represent a potential antimicrobial drug target. We have characterized the kinetic properties of FolD from the Gram-negative pathogen Acinetobacter baumanni and determined high-resolution crystal structures of complexes with a cofactor and two potent inhibitors. The data reveal new details with respect to the molecular basis of catalysis and potent inhibition. A unexpected finding was that our crystallographic data revealed a different structure for LY374571 (an inhibitor studied as an antifolate) than that previously published. The implications of this observation are discussed.", "author" : [{ "dropping-particle" : "", "family" : "Eadsforth", "given" : "Thomas C.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "V.", "family" : "Maluf", "given" : "Fernando", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Hunter", "given" : "William N.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "FEBS Journal", "id" : "ITEM-1", "issue" : "23", "issued" : { "date-parts" : [["2012"]] }, "page" : "4350-4360", "title" : "Acinetobacter baumannii FolD ligand complexes - Potent inhibitors of folate metabolism and a re-evaluation of the structure of LY374571", "type" : "article-journal", "volume" : "279" }, "uris" : ["http://www.mendeley.com/documents/?uuid=39eaedb5-e306-4db7-8969-ab5098477c12"] }, { "id" : "ITEM-2", "itemData" : { "DOI" : "10.1021/acs.jmedchem.5b00687", "ISBN" : "1520-4804 (Electronic)\r0022-2623 (Linking)", "ISSN" : "15204804", "PMID" : "26322631", "abstract" : "\u00a9 2015 American Chemical Society. The bifunctional enzyme N5,N10-methylenetetrahydrofolate dehydrogenase/cyclo hydrolase (FolD) is essential for growth in Trypanosomatidae. We sought to develop inhibitors of Trypanosoma brucei FolD (TbFolD) as potential antiparasitic agents. Compound 2 was synthesized, and the molecular structure was unequivocally assigned through X-ray crystallography of the intermediate compound 3. Compound 2 showed an IC50 of 2.2 \u03bcM, against TbFolD and displayed antiparasitic activity against T. brucei (IC50 49 \u03bcM). Using compound 2, we were able to obtain the first X-ray structure of TbFolD in the presence of NADP+ and the inhibitor, which then guided the rational

design of a new series of potent TbFolD inhibitors.", "author" : [{ "dropping-particle" : "", "family" : "Eadsforth", "given" : "Thomas C.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Pinto", "given" : "Andrea", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Luciani", "given" : "Rosaria", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Tamborini", "given" : "Lucia", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Cullia", "given" : "Gregorio", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Micheli", "given" : "Carlo", "non-dropping-particle" : "De", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Marinelli", "given" : "Luciana", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Cosconati", "given" : "Sandro", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Novellino", "given" : "Ettore", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Presti", "given" : "Leonardo", "non-dropping-particle" : "Lo", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Cordeiro Da Silva", "given" : "Anabela", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Conti", "given" : "Paola", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Hunter", "given" : "William N.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Costi", "given" : "Maria P.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "Journal of Medicinal Chemistry", "id" : "ITEM-2", "issue" : "20", "issued" : { "date-parts" : [["2015"]] }, "page" : "7938-7948", "title" : "Characterization of 2,4-Diamino-6-oxo-1,6-dihydropyrimidin-5-yl Ureido Based Inhibitors of Trypanosoma brucei FolD and Testing for Antiparasitic Activity", "type" : "article-journal", "volume" : "58" }, "uris" : ["http://www.mendeley.com/documents/?uuid=10611627-a00c-4cdf-8f16-edd4306da83e"] }], "mendeley" : { "formattedCitation" : "(Eadsforth, Maluf et al., 2012; Eadsforth et al., 2015)", "plainTextFormattedCitation" : "(Eadsforth, Maluf et al., 2012; Eadsforth et al., 2015)", "previouslyFormattedCitation" : "(Eadsforth, Maluf et al., 2012; Eadsforth et al., 2015)" }, "properties" : { "noteIndex" : 0 }, "schema" : "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }. During this assessment we noted inconsistencies in the Schmidt *et al*. (2000) publication, and in database entries relating to the inhibitors that we sought to understand and/or address. The names and structures of the three

inhibitors together with relevant database codes (PDB code and ligand ID: 1DIA:L24, 1DIB:L34, 1DIG:L37) and used here, are given in Figure 1.

We attempted to use L37 as a lead molecule in inhibitor development (Eadsforth *et al*., 2012a). We repeated the published synthetic route on three occasions, and our analytical data were in excellent agreement with that reported. This extended to the observation that a potent enzyme inhibitor had been prepared. High resolution crystal structures showed however that the inhibitor is not L37 but an isomer. We decided to investigate this discrepancy and noted several issues.

The publication describing the structures of ternary complexes of human DHCH with cofactor NADP and inhibitors L24 (at 2.2 Å resolution), L34 (2.7 Å) or L37 (2.2 Å) showed $2F_o$-F_c electron density maps for L24 and L34 complexes but not for L37. It is widely accepted that such maps are prone to serious model bias and it seemed strange not to present the data for L37, this being after all a highly potent inhibitor. Individual *B*-factors were not refined for the 2.7 Å resolution structure with L34. In all three structures the occupancy of the cofactor atoms and the inhibitors were set at different levels, ranging from 0.1 to 0.5 in order to get *B*-factors comparable to active site residues. The chemical structures of the ligands in the PDB differ from those presented in the publication, an enol form is assigned in the PDB listing with a single bond between C4 O4 and a double bond linking N3 C4. The more reasonable keto form is given for L24 and L34 in the paper and shown in Figure 1. However, the chemical shown in the paper for L37 is inconsistent lacking a ring carbonyl in one figure, but present in another and in the PDB entry.

We next inspected the coordinates. The PDB coordinates for the ligands L24 and L37 have carbon oxygen separations at C4 O4 of around 1.24 Å, appropriate for a double bond and compatible with keto forms, in L34 a C4 O4 distance of 1.39 Å suggests that restraints more appropriate for an enol form may have been used. The model of L37 places two hydrogen bond donor groups, approximately 2.2 Å apart, which is implausible. Moreover, very few contacts exist between the enzyme and L37 which is surprising given the high potency inhibition, K_i 3 nM, reported. The complex with L24 displayed steric clash, i.e short contacts incompatible with established chemical principles, with the cofactor and also with main chain groups in the binding site. On the other hand the complex with L34 had sensible hydrogen bond donor and acceptor groups positioned to interact but with distances 3.5 Å or greater, and non-optimal alignments. We therefore decided to re-assess the three structures after carrying out further refinements.

2.0 Methods

Coordinates and structure factor data for the PDB entries 1DIA, 1DIB and 1DIG were inspected and compared with those output from PDB-REDO, an automated refinement process

designed to improve crystallographic models (Joosten *et al.*, 2009, Touw *et al*., 2016). In common with our previous experience (Rimsa *et al*., 2014, de Souza *et al*., 2017), the PDB-REDO models were identified as being significantly improved, and provided the starting point for further refinement. Although the PDB-REDO automated pipeline had significantly improved model geometry, the assessment and inspection of difference and electron density maps revealed strong features were still present and that the low occupancies of ligands had been retained. PDB-REDO addresses aspects of the protein structure not ligands. We removed the ligands and initiated rounds of electron and difference density map inspection, and refinement. Difference Fourier syntheses were interpreted and then, where justified, ligands were included with full occupancy and, for all three structures, we refined individual *B*-factors with restraints. The geometrical restraints for ligands were generated using the GRADE Web Server v.1.2.13 (www.globalphasing.com, Smart *et al*., 2011). Since these ligands were already included in the PDB chemical components dictionary, the three-letter code for each of them were used as input to run GRADE, which accesses the compound information from RCSB Ligand Expo server and generates the geometry restraints based on the Cambridge Structural Database repository, where available, and uses the quantum mechanics semi empirical molecular orbital model RM1 to generate restraints that are not represented in the CSD (Smart *et al*., 2011). These restraints were then checked to ensure agreement with the structures shown in Figure 1. The water molecules from PDB-REDO were used as a starting point and were inspected. Several were removed and then additional solvent molecules included. Initial inspections revealed that antifolate binding only occurred in one of the two active sites in the asymmetric unit for the complexes with L24 and L34, and overlay of the two polypeptide chains indicated that non-crystallographic symmetry was not strictly maintained in any of the structures hence we did not employ such restraints in refinement. However, as part of the review process we were encouraged to investigate the effect of the use of non-crystallographic symmetry restraints in the form of local structure symmetry restraints. This reduced the R_{free} values we had obtained and a further comment on this subject is made below. Model manipulation and map inspections were performed using COOT (Emsley & Cowtan, 2004) and least-squares refinement calculations used REFMAC {ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1107/S0907444904019158", "ISBN" : "0907-4449 (Print)\\n0907-4449 (Linking)", "ISSN" : "09074449", "PMID" : "15572765", "abstract" : "CCP4mg is a project that aims to provide a general-purpose tool for structural biologists, providing tools for X-ray structure solution, structure comparison and analysis, and publication-quality graphics. The map-fitting tools are available as a stand-alone package, distributed as 'Coot'.", "author" : [{ "dropping-particle" : "", "family" : "Emsley", "given" : "Paul", "non-

dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Cowtan", "given" : "Kevin", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "Acta Crystallographica Section D: Biological Crystallography", "id" : "ITEM-1", "issue" : "12 I", "issued" : { "date-parts" : [["2004"]] }, "page" : "2126-2132", "publisher" : "International Union of Crystallography", "title" : "Coot: Model-building tools for molecular graphics", "type" : "article-journal", "volume" : "60" }, "uris" : ["http://www.mendeley.com/documents/?uuid=6a3ed0c2-1609-4088-a8ed-0318d8a63f27"] }, { "id" : "ITEM-2", "itemData" : { "DOI" : "10.1107/S0907444911001314", "ISBN" : "0907444911001", "ISSN" : "09074449", "PMID" : "21460454", "abstract" : "This paper describes various components of the macromolecular crystallographic refinement program REFMAC5, which is distributed as part of the CCP4 suite. REFMAC5 utilizes different likelihood functions depending on the diffraction data employed (amplitudes or intensities), the presence of twinning and the availability of SAD/SIRAS experimental diffraction data. To ensure chemical and structural integrity of the refined model, REFMAC5 offers several classes of restraints and choices of model parameterization. Reliable models at resolutions at least as low as 4\u2005\u00c5 can be achieved thanks to low-resolution refinement tools such as secondary-structure restraints, restraints to known homologous structures, automatic global and local NCS restraints, `jelly-body' restraints and the use of novel long-range restraints on atomic displacement parameters (ADPs) based on the Kullback-Leibler divergence. REFMAC5 additionally offers TLS parameterization and, when high-resolution data are available, fast refinement of anisotropic ADPs. Refinement in the presence of twinning is performed in a fully automated fashion. REFMAC5 is a flexible and highly optimized refinement package that is ideally suited for refinement across the entire resolution spectrum encountered in macromolecular crystallography.", "author" : [{ "dropping-particle" : "", "family" : "Murshudov", "given" : "Garib N.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Skub\u00e1k", "given" : "Pavol", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Lebedev", "given" : "Andrey a.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Pannu", "given" : "Navraj S.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Steiner", "given" : "Roberto a.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Nicholls", "given" : "Robert a.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Winn", "given" : "Martyn D.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Long", "given" : "Fei", "non-dropping-

particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Vagin", "given" : "Alexei a.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "Acta Crystallographica Section D: Biological Crystallography", "id" : "ITEM-2", "issue" : "4", "issued" : { "date-parts" : [["2011"]] }, "page" : "355-367", "title" : "REFMAC5 for the refinement of macromolecular crystal structures", "type" : "article-journal", "volume" : "67" }, "uris" : ["http://www.mendeley.com/documents/?uuid=94c35ed1-a520-4d5f-b8be-e4b014c2960d"] }], "mendeley" : { "formattedCitation" : "(Emsley & Cowtan, 2004; Murshudov et al., 2011)", "plainTextFormattedCitation" : "(Emsley & Cowtan, 2004; Murshudov et al., 2011)", "previouslyFormattedCitation" : "(Emsley & Cowtan, 2004; Murshudov et al., 2011)" }, "properties" : { "noteIndex" : 0 }, "schema" : "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }**}**. Model geometry was assessed using MolProbity **{**ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1107/S0907444909042073", "ISBN" : "0907-4449", "ISSN" : "09074449", "PMID" : "20057044", "abstract" : "MolProbity is a structure-validation web service that provides broad-spectrum solidly based evaluation of model quality at both the global and local levels for both proteins and nucleic acids. It relies heavily on the power and sensitivity provided by optimized hydrogen placement and all-atom contact analysis, complemented by updated versions of covalent-geometry and torsion-angle criteria. Some of the local corrections can be performed automatically in MolProbity and all of the diagnostics are presented in chart and graphical forms that help guide manual rebuilding. X-ray crystallography provides a wealth of biologically important molecular data in the form of atomic three-dimensional structures of proteins, nucleic acids and increasingly large complexes in multiple forms and states. Advances in automation, in everything from crystallization to data collection to phasing to model building to refinement, have made solving a structure using crystallography easier than ever. However, despite these improvements, local errors that can affect biological interpretation are widespread at low resolution and even high-resolution structures nearly all contain at least a few local errors such as Ramachandran outliers, flipped branched protein side chains and incorrect sugar puckers. It is critical both for the crystallographer and for the end user that there are easy and reliable methods to diagnose and correct these sorts of errors in structures. MolProbity is the authors' contribution to helping solve this problem and this article reviews its general capabilities, reports on recent enhancements and usage, and presents evidence that the resulting improvements are now beneficially affecting the global database.", "author" : [{ "dropping-particle" : "", "family" : "Chen", "given" : "Vincent B.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Arendall", "given" : "W. Bryan", "non-dropping-particle" :

"", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Headd", "given" : "Jeffrey J.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Keedy", "given" : "Daniel A.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Immormino", "given" : "Robert M.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Kapral", "given" : "Gary J.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Murray", "given" : "Laura W.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Richardson", "given" : "Jane S.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Richardson", "given" : "David C.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "container-title" : "Acta Crystallographica Section D: Biological Crystallography", "id" : "ITEM-1", "issue" : "1", "issued" : { "date-parts" : [["2010"]] }, "page" : "12-21", "publisher" : "International Union of Crystallography", "title" : "MolProbity: All-atom structure validation for macromolecular crystallography", "type" : "article-journal", "volume" : "66" }, "uris" : ["http://www.mendeley.com/documents/?uuid=9584c613-fb1d-4d1c-b5b7-34b89c826b41"] }], "mendeley" : { "formattedCitation" : "(Chen et al., 2010)", "plainTextFormattedCitation" : "(Chen et al., 2010)", "previouslyFormattedCitation" : "(Chen et al., 2010)" }, "properties" : { "noteIndex" : 0 }, "schema" : "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" } and the PDB validation server (validate-rcsb-2.wwpdb.org). All coordinates were checked against the structure factor data using the sf-tool web server (sf-tool.wwwpdb.org), the density correlation was calculated using MAPMAN (Jones and Thirup, 1986) and REFMAC. The models were deposited in the PDB. Figures were generated by Pymol (v.1.3, {ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "author" : [{ "dropping-particle" : "", "family" : "Schrodinger", "given" : "LLC", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }], "id" : "ITEM-1", "issued" : { "date-parts" : [["0"]] }, "title" : "The Pymol Molecular Graphics System, Version 1.3", "type" : "article" }, "uris" : ["http://www.mendeley.com/documents/?uuid=56dd8fe8-ca50-423a-a75f-7ceaee007cc6"] }], "mendeley" : { "formattedCitation" : "(Schrodinger)", "manualFormatting" : "(Schrodinger, LLC)", "plainTextFormattedCitation" : "(Schrodinger)", "previouslyFormattedCitation" : "(Schrodinger)" }, "properties" : { "noteIndex" : 0 }, "schema" : "https://github.com/citation-style-language/schema/raw/master/csl-citation.json" }. The crystallographic statistics for the original PDB entries, the PDB-REDO starting model and arising from this study are given in supplementary material, Tables S1, S2, S3 for 1DIA, 1DIB, 1DIG respectively.

3.0 Results and Discussion

3.1 Overall structure and initial comments

Three isomorphous, medium resolution crystal structures of human DHCH were determined in space group $P2_12_12_1$ with a dimer in the asymmetric unit {ADDIN CSL_CITATION { "citationItems" : [{ "id" : "ITEM-1", "itemData" : { "DOI" : "10.1021/bi992734y", "ISBN" : "0006-2960 (Print)\\r0006-2960 (Linking)", "ISSN" : "00062960", "PMID" : "10828945", "abstract" : "Enzymes involved in tetrahydrofolate metabolism are of particular pharmaceutical interest, as their function is crucial for amino acid and DNA biosynthesis. The crystal structure of the human cytosolic methylenetetrahydrofolate dehydrogenase/cyclohydrolase (DC301) domain of a trifunctional enzyme has been determined previously with a bound NADP cofactor. While the substrate binding site was identified to be localized in a deep and rather hydrophobic cleft at the interface between two protein domains, the unambiguous assignment of catalytic residues was not possible. We succeeded in determining the crystal structures of three ternary DC301/NADP/inhibitor complexes. Investigation of these structures followed by site-directed mutagenesis studies allowed identification of the amino acids involved in catalysis by both enzyme activities. The inhibitors bind close to Lys56 and Tyr52, residues of a strictly conserved motif for active sites in dehydrogenases. While Lys56 is in a good position for chemical interaction with the substrate analogue, Tyr52 was found stacking against the inhibitors' aromatic rings and hence seems to be more important for proper positioning of the ligand than for catalysis. Also, Ser49 and/or Cys147 were found to possibly act as an activator for water in the cyclohydrolase step. These and the other residues (Gln100 and Asp125), with which contacts are made, are strictly conserved in THF dehydrogenases. On the basis of structural and mutagenesis data, we propose a reaction mechanism for both activities, the dehydrogenase and the cyclohydrolase.", "author" : [{ "dropping-particle" : "", "family" : "Schmidt", "given" : "Andrea", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Wu", "given" : "Haiping", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Mackenzie", "given" : "Robert E.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Chen", "given" : "Victor J.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Bewly", "given" : "Jesse R.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Ray", "given" : "James E.", "non-dropping-particle" : "", "parse-names" : false, "suffix" : "" }, { "dropping-particle" : "", "family" : "Toth", "given" : "John E.", "non-dropping-particle" : "", "parse-names" : false, "suffix"

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3.1 The cofactor

In PDB entries 1DIA, 1DIB and 1DIG the NADP occupancies had been adjusted to values lower than one. We obtained a satisfactory refinement using full occupancy and this improved the density correlation (Table S4), and resulted in acceptable *B*-factors that are comparable with the overall B-factors of the protein atoms (Supporting Information, Tables S1-3). The placement and conformation of the cofactors is not significantly different from those previously reported.

3.2 L34

Despite this structure being associated with the lowest resolution data set, 2.7 Å, different Fourier syntheses were unambiguous (e.g. Figure 1). Density for the ligand is well defined and it refined

satisfactorily with full occupancy. We, appear to be more conservative than the original authors and included fewer water molecules in the model. The density correlation coefficient increased from 0.77 to 0.87, but R_{free} increased from 0.241 to 0.260 when the refinement, which did not employ non-crystallographic symmetry restraints, converged. The inclusion of local structure symmetry restraints then reduced the R_{free} to 0.247. The R_{free} values for the original structure and the PDB-REDO model were 0.240 and 0.241, respectively. The model displays improved geometry for chemical interactions in the active site with the ligand now positioned closer to key residues (Figure 2). The L34 N1 and N8 distances to Leu101 N and O are reduced to 3.2 Å and 3.0 Å from 3.7 Å and 3.5 Å, an appropriate separation for hydrogen bonding interactions not described previously. In addition, our model now has both oxygen atoms on the side chain of Asp125 forming hydrogen-bonding interactions with L34. The general features of enzyme-ligand interactions remain similar and consistent with the observation that L34 is a potent inhibitor of the enzyme with K_i of 30 nM (Schmidt *et al*., 2000).

3.3 L24

Here, although Fourier syntheses indicate that a ligand may be present in the active site, they do not identify a single well-defined pose and features in difference maps following the refinement of different poses suggested that two orientations might be present, one of which was identified in the original work (Figure 1). We refined two poses of L24 each with occupancy 0.5. We note that the overall L24 B-factor for the two orientations are approximately twice and five times that of the overall B-factor for protein atoms. In both orientations the aromatic head group is positioned further out of the binding site compared to L34. A lack of interactions with the protein would be in agreement with the observation that L24 is only a weak inhibitor with K_i around 30 μM (Schmidt *et al*., 2000). Although our refinement has reduced the steric clashes present in the entry 1DIA, we suggest that further experimental work concentrating on sample preparation might be required to pursue an improved structure of this inhibitor in complex with the enzyme.

3.4 L37

This molecule was reported to possess a K_i against the human enzyme of about 3 nM (Schmidt *et al*., 2000). We previously raised questions about the assignment of the chemical structure of L37, a compound that has been patented (company code: LY374571) and which progressed into clinical trials. We made three attempts to repeat the synthesis of L37 using the published protocol, and succeeded only in obtaining an isomer (PDB ligand code: 9L9). It is possible that, for reasons

unknown, we were unable to repeat the reported synthesis. An alternative explanation is that the original researchers did not prepare L37 but rather they themselves first synthesized the isomer. The analytical data reported for the isomers does not distinguish between them and we were only alerted to the discrepancy when we determined a high-resolution crystal structure (Eadsforth *et al*., 2012). A reason to further investigate the published L37 complex was to seek out data in support of the L37 structure. However, the question as to whether this molecule has actually been synthesized and characterized remains open. Irrespective of that, our interpretation of the human enzyme crystallographic data is that no well-ordered inhibitor, neither L37 or 9L9, is present in the structure and the data then represents a binary complex with the cofactor NADP. We placed a number of water molecules in the active site. Some of these match to the positions of potential hydrogen bonding functional groups of the putative ligand in the entry 1DIG.

4.0 Conclusions

The PDB is an extremely valuable resource for biochemical and medicinal chemistry research (Burley *et al*., 2017) but unfortunately, serious errors in ligand-protein complexes have been reported (Wlodawer *et al*., 2018). For our own part, we previously showed that in structures of human peroxisome proliferator-activated receptors-β/δ the fatty acid binding site is occupied by endogenous ligands derived from the bacterial expression system and not lipid-lowering synthetic agents (Fyffe *et al*., 2006). In the glutaminase domain of *Trypanosoma brucei* CTP synthetase we corrected the stereochemistry of the inhibitor acivicin, which completely changes the description of interactions that stabilize the covalent complex (de Souza *et al.,* 2017). In the case discussed here, of three DHCH ligand complexes, we have deposited models in the PDB following further refinement and with our interpretation of the data. They are therefore available to researchers with interests in development of antifolates. Perhaps of most value will be the negative result relating to L37. Since the placement of the potent inhibitor reported in PDB entry 1DIG is not substantiated by the crystallographic data then that structure should not be used to guide ligand development.

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Figure legends

Figure 1. The structures and names of DHCH inhibitors discussed in this work. A. L34 is (2S)-2-[[4-[(6aR)-3-amino-1,9-dioxo-5,6,6a,7-tetrahydro-4H-imidazo[1,5-f]pteridin-8-yl]benzoyl]amino]pentanedioic acid. L24 is (2R)-2-[[4-[2-[(6R)-2-amino-4-oxo-5,6,7,8-tetrahydro-1H-pyrido[2,3-d]pyrimidin-6-yl]ethyl]benzoyl]amino]pentanedioic acid. L37 is (2R)-2-[[4-[(2,5-diamino-4-oxo-1H-pyrimidin-6-yl)carbamoylamino]benzoyl]amino]pentanedioic acid. **B.** Omit Fo-Fc Polder maps (Liebschner *et al.,* 2017) contoured at 3.0 r.m.s.d. after additional refinements. These omit maps were generated using the observed structure factors Fo of each model, and the calculated structure factors Fc generated after setting zero occupancies to the inhibitors, and removing the bulk solvent correction. The omit map for 1ECQ is presented with the L24 pteridine ring in two orientations: white carbon sticks represents L24 pteridine ring in the

same orientation as presented in 1DIA and pink carbon sticks represents the pteridine ring 180° rotated in relation to 1DIA. For L37, the coordinates from PDB entry 1DIA are shown.

Figure 2. Potential hydrogen bonding interactions between the inhibitor L34 and Lys56, Leu101, Asp125 and Gly273. For comparative purposes, the models 6ECQ and 1DIB are superimposed. Carbon positions associated with 6ECQ are colored grey, those with 1DIB brown. All oxygen and nitrogen positions are red and blue respectively. Distance measurements (in Å) are represented as black dashed lines.